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(54) Title: SYNTHESIS OF FLUORESCENCE-LABELLED NUCLEIC ACIDS

(57) Abstract

The present invention provides a method for synthesizing nucleic acids of at least 200 or 500 bases length, including strands of up to several kb in length, wherein at least one of the four NTPs is completely substituted by a fluorescent-labelled ribo- or deoxyribonucleotide, r- or dNTP-X. The method includes the use of dNTP-X compounds having the nucleotide base moiety covalently joined to the fluorophore by a linker chain of 8 to 12 atoms length, the use of a processive DNA polymerase, and the use of single-strand DNA binding protein in the reaction mixture. Novel compounds suitable for use in synthesizing fully labelled fluorescent DNA and a kit for carrying out the method are also disclosed. The method of the present invention is useful to provide fully labelled fluorescent RNA or DNA for sequence analysis, for histochemical fluorescent labelling and for micro-analytical techniques where highly fluorescent RNA or DNA of specified sequence is desired.

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SYNTHESIS OF FLUORESCENCE-LABELLED NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

This is a continuation-in-part of application Serial No. 07/852,705, filed March 17, 1992. The field of the invention is enzyme-catalyzed DNA synthesis particularly synthesis of fluorescence-labelled DNA. More particularly, the invention relates to synthesis of fluorescence-labelled nucleic acids having sequence complementary to a specified nucleic acid template.

Techniques for labelling nucleic acids with a fluorescence marker can be divided into two categories: post-synthetic chemical or enzymatic modification, and incorporation of fluorescence-labelled precursors during synthesis. For examples of the former category see Dirks, R.W. et al. (1991) Exptl. Cell Res. 194:310-315; Lichter, P. et al. (1988) Human Genetics 80:224-234; and Lichter, P. et al. (1990) Proc. Natl. Acad. Sci. USA 87:6634-6638. The present invention belongs in the latter category. A typical enzyme-catalyzed DNA synthesis reaction employs a template DNA or RNA, a primer oligonucleotide having a sequence complementary to a segment of the template DNA, an enzyme catalyst and four deoxynucleotide (dNTP) precursors, dATP, dGTP, dCTP and dTTP or, alternatively, dUTP. Various enzyme catalysts are known in the art, such as *E. coli* DNA polymerase, T7 DNA polymerase, Klenow fragment of DNA polymerase, Taq DNA

polymerase, reverse transcriptase and the like. The enzymes can be characterized by the degree to which an enzyme molecule remains continuously associated with the same growing strand of nascent DNA. Those enzymes which tend to remain associated with the same nascent DNA strand for longer times are termed "processive." In general, the term "processivity" refers to the number of nucleotides that a DNA polymerase incorporates during DNA synthesis before dissociating from the template-primer complex. In the strictest sense, a "processive" enzyme incorporates more than one nucleotide before dissociating and a non-processive (or distributive) polymerase dissociates from the template-primer after every addition of a new nucleotide. The concepts of "processivity" and "high processivity" are discussed in A. Kornberg and T. Baker, DNA Replication (2nd Ed., W.H. Freeman and Co., N.Y., 1992) p. 494.

Enzyme catalyzed RNA synthesis proceeds along similar lines although template and primer requirements are somewhat different, and riboNTP's are employed as precursors, all as well-known in the art. Typical RNA polymerases include bacterial RNA polymerases, eucaryotic RNA polymerases I, II and III, and RNA replicases, for example, Qβ replicase.

Fluorescence-labelling has been accomplished in the prior art by partially substituting a fluorescence-labelled NTP analog so that during synthesis an analog molecule occasionally replaces a normal NTP precursor in the sequence. Various fluorescent dNTP derivatives are known in the art, having a fluorophore, such as fluorescein or rhodamine, covalently linked to the purine or pyrimidine base by a linker group. Examples of prior art fluorescent dNTP derivatives are Rhodamine-6-dATP, Rhodamine-6-dCTP, Fluorescein-7-dUTP and Fluorescein-12-dUTP. The latter is available from Boehringer Mannheim Biochemica.

The fluorescent NTPs used in the prior art have been considered likely to interfere with synthesis due to the steric interference of the large fluorophore moiety. For synthesis of

DNA longer than 200 bases fluorophore-substituted dNTPs have been employed as partial substituents for the normal dNTP, i.e., the synthesis reaction mixture contains less than 1.0 mole fraction of total dNTP as its fluorescent derivative. For example, a typical reaction mixture might contain a 1:3 mole ratio of Fl-12-dCTP:dCTP.

Newer methods of DNA sequence analysis have generated a need for DNA having at least one of the four dNTPs completely substituted by a fluorescence-labelled dNTP (dNTP-X). For example, Jett et al. U.S. patent no. 4,962,037 have developed techniques for sequentially analyzing the exonuclease products of a single DNA molecule. Each deoxynucleotide labelled with a fluorophore is detected in sequence as it is released by exonuclease action, using a sensitive flow-fluorometric technique. The efficiency of the foregoing technique will be improved by providing fully fluorescent labelled DNA of greater length than available heretofore.

Methods for synthesizing DNA having at least one dNTP fully substituted by a fluorescent dNTP (dNTP-X) have been hampered by the steric hindrance associated with incorporating such derivatives into a DNA molecule. Synthesis of DNA strands of up to 500 bases in length has been reported using dNTP-X having a linker of less than 8 atoms length between the nucleotide base moiety and the fluorophore. (Jett, et al., U.S. Application Serial No. 07/765,277.)

SUMMARY OF THE INVENTION

The invention provides a method for synthesizing nucleic acids wherein at least one of the four NTPs is completely substituted by a fluorescent-labelled ribo- or deoxyribonucleotide, r- or dNTP-X. In one embodiment, labelled DNA of at least 200 bases length can be synthesized. In a preferred embodiment, labelled DNA of at least 500 bases length can be synthesized. The method includes the use of dNTP-X

compounds having the nucleotide base moiety covalently joined to the fluorophore by a linker chain of 8 to 12 atoms length. In the preferred embodiment, such linker chains are those lacking an ether linkage. In another preferred embodiment, a processive DNA polymerase is employed. In another preferred embodiment, single-strand DNA binding protein is provided in the reaction mixture.

Novel compounds suitable for use in synthesizing fully labelled fluorescent DNA are disclosed herein. These include Rho-8-dCTP, Rho-10(J)-dCTP, Fl-10(J)-dCTP, Rho-15-dCTP, Fl-15-dCTP, Rho-12-dUTP, Fl-12-dUTP, Fl-8-dATP, Rho-8-dATP, Fl-15-dATP, Res-10-dUTP, HC-6-dUTP, and Rho-12-dUTP (R). In preferred compounds of the present invention, linkage of the fluorophore does not modify a site on the purine or pyrimidine base that is normally involved in the hydrogen bonding interactions of base-pairing. By combining a preferred fluorescent dNTP, single-strand binding protein and a preferred polymerase, the method of the present invention has successfully produced fully single-labelled fluorescent DNA of greater than 7000 bases length.

The method of the invention is useful to provide fully labelled fluorescent RNA or DNA for sequence analysis, for histochemical fluorescent labelling and for micro-analytical techniques where highly fluorescent RNA or DNA of specified sequence is desired. A useful kit for carrying out the method of the invention is also provided.

DETAILED DESCRIPTION OF THE INVENTION

The method of the invention uses conventional enzyme-catalyzed RNA or DNA synthesis reactions in which the complementary sequence of a template nucleic acid strand is synthesized in a reaction mixture containing an RNA or DNA polymerase enzyme, four ribo or deoxy nucleotide triphosphates (r- or dNTPs) and optionally an oligonucleotide primer, depending on the specific requirements of the enzyme chosen to catalyze synthesis. The template nucleic acid is usually cloned or

purified DNA or RNA whose sequence is to be determined, or for which a fluorescent complement is desired. The template DNA is usually in single-stranded, denatured, partially single-stranded, or partially denatured form depending on the template requirements of the polymerase employed. Primer DNA is typically an oligonucleotide whose sequence is complementary to a segment of the template nucleic acid. Synthesis is considered to proceed by stepwise addition of ribo- or deoxynucleotides to the 3′-end of the primer, thereby extending the length of the primer, with concomitant release of a pyrophosphate from the precursor r- or dNTP. The order of addition of r- or dNTPs is dictated by the template sequence, such that the newly synthesized RNA or DNA has a sequence complementary to the template sequence according to the known base-pairing relationships, A with T (or U) and G with C, of nucleic acids.

Enzymes which catalyze DNA synthesis are termed DNA polymerases. Many DNA polymerases are known, for example T5 polymerase (Chatterjee, D., U.S. Patent 5,047,342), T7 polymerase, E. coli polymerase Klenow fragment, Taq polymerase, Vent™ (New England Biolabs) polymerase and PRD1 polymerase(Savilahti, H. et al. (1991) J. Biol. Chem. 266:18737-18744). Reverse transcriptases, which catalyze DNA synthesis using a RNA template, are also suitable for the present invention. A wide selection of such enzymes is commercially available, including AMV-RT and M-MLV-RT (GIBCO BRL), and HIV-RT. Highly processive enzymes are preferred herein. A "highly processive" enzyme is herein defined as one that incorporates 50 or more nucleotides before dissociating from the template-primer complex under a given set of reaction conditions. Highly processive DNA polymerases include phage T5 polymerase and derivatives thereof ("T5"), phage T7 polymerase and derivatives thereof ("T7"), phage Phi-29-type polymerases ("Phi-29"), and *E. coli* pol III holozyme. T7 DNA polymerase (also called "T5 gene 5 protein") by itself is a DNA polymerase with low processivity. In the presence of thioredoxin cofactor, however, T7 becomes highly processive, incorporating thousands of nucleotides from

a given primer without dissociation (S. Tabor et al. [1987] J. Biol. Chem. 262:16212; S. Tabor and C. Richardson, U.S. Patent 4,795,699). As used herein, the Phi-29-type polymerases include Phi-29, Cp-1, PRD1, Phi-15, Phi-21, PZE, PZA, Nf, M2Y, BIO3, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, AND L17 (Blanco et al., U.S. Patent 5,001,050). E. coli pol III holozyme is a highly processive enzyme (processivity value greater than 5000), as described by A. Kornberg and T. Baker in DNA Replication (2nd Ed., W.H. Freeman and Co., NY, 1992), at pages 494-495. Commercially available RNA polymerases include SP6 RNA polymerase, T3 and T7 RNA polymerases.

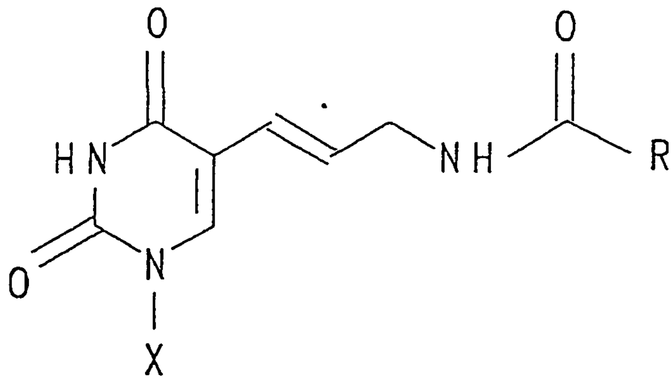
In the synthesis of unlabeled DNA, the four precursor dNTPs are dATP, dGTP, dCTP, and dTTP or, alternatively, dUTP, usually provided in approximately equimolar amounts with one another. Either dTTP or dUTP can be used in a DNA polymerase-catalyzed synthesis of DNA, and for the purposes of the present invention dTTP and dUTP can, as a practical matter, be used interchangeably, as can their respective fluorescence-labelled derivatives. In prior art fluorescence-labelling reactions, one of the dNTPs is partially substituted by a fluorescence-labelled derivative. For example, the mole ratio of dTTP or dUTP to Fl-dUTP would typically be about 2:1. As a result, the reaction product would contain Fl-dU in some sequence loci where dT or dU would be incorporated. However, the ratio of Fl-dU to dT or dU in the product might be lower than that of the reaction mixture because reaction kinetics would favor incorporation of dT or dU over the derivative.

Fluorescence-labelled r- or dNTP derivatives are herein abbreviated r- or dNTP-X. The r- or dNTP-X compounds of the present invention have two basic components, a fluorophore and a linker. The fluorophore can be any highly fluorescent compound, including, without limitation, fluorescein, rhodamine, resorufin, and hydroxycoumarin. A variety of fluorophores having different excitation and emission maxima are desirable, in applications where more than one r- or dNTP is labelled. The

linker is typically a chain of greater than 7 and preferably 8 to 12 atoms covalently joining the purine or pyrimidine base of the r- or dNTP to the fluorophore. When the fluorophore is resorufin or hydroxycoumarin, the linker can be shorter, preferably 5 atoms length. The linker can be an aliphatic chain of C-C bonds, optionally combined with alkene groups, amide bonds, ether groups and the like. However, for synthesis of DNA of greater than 500 bases length, r- or dNTP-X compounds lacking ether groups in the linker are preferred. It is preferred that the linker joins the purine or pyrimidine moiety at a site or atom not involved in the hydrogen bond formation of nucleotide base pairing. For adenine, these sites are the 6-amino group and the 1-N of the purine ring; for guanine, the 6-OH , the 1-N and the 2-amino group; for cytosine, the 4-amino group, the 3-N and the 2-OH; and for thymine, the 4-oxo group and the 3-N.

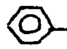
The structures of dNTP-X compounds useful in the practice of the synthesis method are shown in Tables 1-4. The nomenclature used herein identifies the fluorophore, the linker length (except in the case of HC-6-dUTP and Res-10-dUTP) and the nucleotide base. HC-6-dUTP and Res-10-dUTP, as used herein, refer to the commercial names for hydroxy-coumarin-6-dUTP and resorufin-10-dUTP, respectively. Abbreviations for fluorescein, rhodamine, resorufin, and hydroxy-coumarin are Fl, Rho, Res, and HC, respectively. For example, Rho-12-dUTP is dUTP joined to rhodamine by a linker chain of 12 atoms. Use of a Jeffamine precursor for synthesis is indicated by including "(J)" in the abbreviated name and also indicates the presence of ether groups in the linker. Use of an "(R)" immediately after the abbreviated name indicates a rigid linker as compared to the non-rigid analog; e.g., Rho-12-dUTP(R) versus Rho-12-dUTP. Specific fluorescence-labelled nucleotides are designated herein as dATP-X, dGTP-X, dCTP-X and dUTP-X.

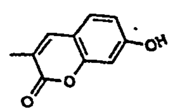
TABLE 1

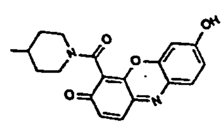


Rho-5-dUTP: R = TETRAMETHYL-RHODAMINE

Rho-12-dUTP: R = $(CH_2)_5$-NH-CO-TETRAMETHYL-RHODAMINE

Rho-12-dUTP(R): R = -CH_2-NH-$(CH_2)_2$-NH-CO-TETRAMETHYL-RHODAMINE

HC-6-dUTP: R = 

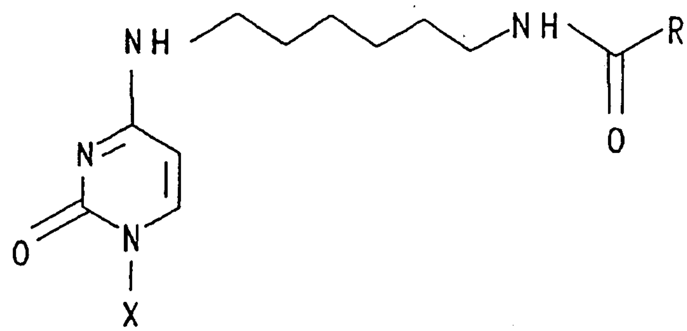
Res-10-dUTP: R = 

Fl-5-dUTP: R = FLUORESCEIN

Fl-12-dUTP: R = $(CH_2)_5$-NH-CO-FLUORESCEIN

X = DEOXYRIBOSE-5'-TRIPHOSPHATE

TABLE 2



Rho-8-dCTP: R = TETRAMETHYL-RHODAMINE

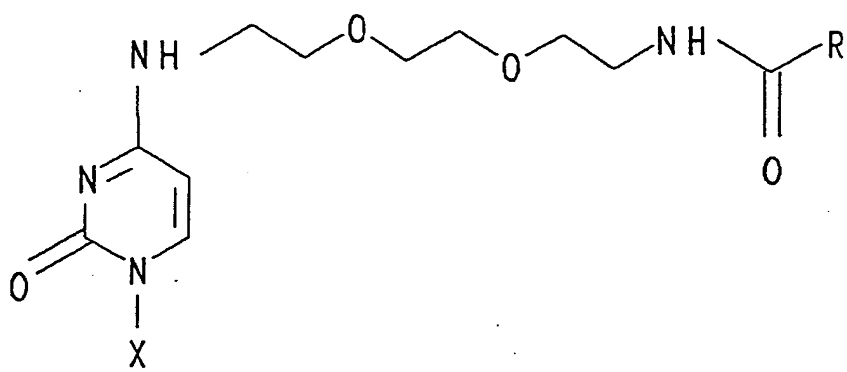
Fl8-dCTP: R = FLUORESCEIN

Rho-15-dCTP: R = $-(CH_2)_5$NH-CO-TETRAMETHYL-RHODAMINE

Fl 15-dCTP: R = $-(CH_2)_5$NH-CO-FLUORESCEIN

X = DEOXYRIBOSE-5'-TRIPHOSPHATE

TABLE 3

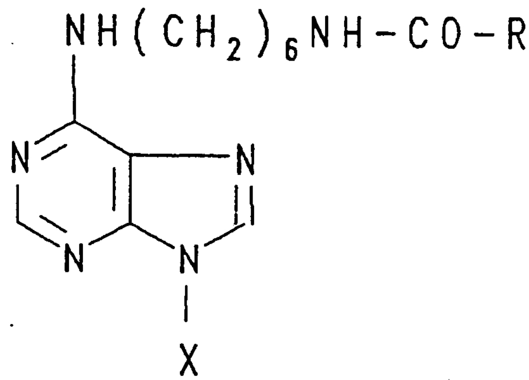


Rho-10(J)-dCTP: R = TETRAMETHYL-RHODAMINE

Fl-10(J)-dCTP: R = FLUORESCEIN

X = DEOXYRIBOSE-5′-TRIPHOSPHATE

TABLE 4



Rho-8-dATP: R = TETRAMETHYL-RHODAMINE

Fl-8-dATP: R = FLUORESCEIN

Fl-15 dATP: R = $(CH_2)_5$-NH-CO-FLUORESCEIN

X = DEOXYRIBOSE-5′-TRIPHOSPHATE

Because of the prior art practice of partially substituting a dNTP-X for the corresponding dNTP in a DNA synthesis reaction, specific distinguishing terminology is used herein. Complete substitution for one of the r- or dNTPs means that one of the r- or dNTPs is replaced entirely by an r- or dNTP-X. For example, complete substitution of dCTP by dCTP-X means that the reaction mixture contains dATP, dGTP, dUTP and dCTP-X, but no dCTP (within the practical limitations imposed by the purity of the dCTP-X preparation). The DNA synthesized in such a reaction mixture is termed herein fully single nucleotide labelled DNA. Every cytosine (C) residue in such DNA would be replaced by C-X, a fluorescence-labelled cytosine, except for that portion represented by the original primer. Fully double nucleotide labelled DNA is then DNA in which two of the four dNTPs are completely substituted for by their corresponding dNTP-X derivatives. Similarly for fully triple and quadruple nucleotide-labelled DNA.

Addition of single-strand binding protein to the reaction mixture has been found to enhance yield and to increase the maximum length of fully labelled product. A total yield increase of over 200% was obtained in some instances.

Complete substitution of a dNTP-X results in inhibition of the DNA synthesis reaction. The extent of inhibition varies with the polymerase used and the dNTP-X. Inhibition from 40% to 99% relative to control reactions using the unmodified dNTPs was observed. In general reactions inhibited more than 95% failed to yield fully labelled DNA of greater than 500 bases length. The extensive inhibition observed with complete substitution may have been a significant factor in directing the art away from complete substitution heretofore.

A kit combining the main components for carrying out the invention is also provided. The kit includes a DNA polymerase enzyme and an assortment of dNTPs and dNTP-X substrates. In one embodiment a kit designed for complete substitution of one dNTP-X

will contain 3 dNTPs and one dNTP-X, either separately packaged or premixed. In another embodiment, providing greater flexibility, a complete set of dNTPs and dNTP-X derivatives is provided, to facilitate any desired combination of complete single, double or multiple fluorescence labelling. Such kits optionally provide a standard template DNA and standard primer for control and calibration, however, the end user will provide the desired template and primer for the specific desired purpose.

The novel compounds of the invention and the method of DNA synthesis using said compounds, or known compounds having the described characteristics, is described in the following examples. All methods and procedures not otherwise described herein were carried out using published methods, as referenced, or by techniques well-known to those of ordinary skill in the art. Standard abbreviations are used, unless otherwise noted. The principles and teachings disclosed herein with respect to DNA synthesis apply also to RNA synthesis, with appropriate modifications of reaction conditions, adapted to RNA synthesis and RNA synthesis-catalyzing enzymes, as are well-known in the art.

EXAMPLES

Example 1: Synthesis of Modified Nucleoside Triphosphates

1. Amino-nucleoside triphosphates.

N^4-Aminohexyl dCTP (precursor of Rho-8-dCTP, see Table 5) and N^4-Jeffamine-dCTP (precursor of Rho-10(J)-dCTP and Fl-10(J)-dCTP) were prepared by the transamination method of Draper (1984) Nucl. Acids Res. 12:989-1003.

N^6-Aminohexyl dATP (precursor of Fl-8-dATP and Rho-8-dATP) was prepared by the method of Gebeyehu et al. (1987) Nucl. Acids Res. 15:4513-4534.

Allylamine dUTP (precursor of Fl-12-dUTP, Rho-12-dUTP and Rho-4-dUTP) was prepared according to Langer et al. (1981) Proc. Natl. Acad. Sci USA 78:6633-6637.

2. Labelling of amino-nucleoside triphosphates with fluorescent dyes.

The amino-nucleoside triphosphates (10-20 μmol) were dissolved in sodium bicarbonate (0.4 M, 500 μl) or sodium borate solution (0.1 M) and treated with a 3 to 5-fold molar excess of the N-hydroxysuccinimide ester of the dye in anhydrous dimethyl formamide (500 μl). The mixture was reacted for 3 - 18 hr. at room temperature. The reaction was monitored by thin layer chromatography (silica gel; butanol: acetone: acetic acid: 5% ammonium hydroxide: water/70:50:30:30:20) and/or by HPLC. The crude mixtures were diluted in water (200 - 300 ml), loaded on a 10 - 15 cm long, by 1 cm diameter column of mild anion exchange resins and eluted, sequentially, with 0.01, 0.2 and 0.5 M triethylammonium bicarbonate until the fraction containing the fluorescent dNTP was collected. After desalting of the appropriate column fraction, TLC, HPLC and capillary electrophoresis analysis was used to assess the purity and characteristic elution patterns of the desired product. The compounds were characterized by their U.V. spectra as the overlapping spectra of the starting amino modified base and the dyes. Yields of fluorescent nucleotides were 50 - 60%.

TABLE 5 : SIZE OF FLUORESCENT DNAS SYNTHESIZED IN OVERNIGHT REACTIONS AS ASSAYED BY ALKALINE AGAROSE GEL ELECTROPHORESIS

dNTP	T5 (Exo^-)	T7 (Exo^-)	Klenow	TAQ	Vent	Vent (Exo)	PRD1
Rho-8-dCTP	500-5000	500-2000	500-2000	200-300	200-500	200-500	ND
Rho-10(J)-dCTP	200-500	200-300	<200	<200	<200	200-500	ND
Fl-10(J)-dCTP	200-500	200-300	ND	ND	ND	ND	ND
Green-dCTP	300-700	200-300	200-500	200-300	<200	<200	ND
Orange-dCTP	200-500	200-300	200-300	200-300	<200	200-300	ND
Fl-12-dUTP	500-7000	500-4000	500-1000	500-1500	500-3000	500-4000	200-5000
Rho-12-dUTP	500>7000	500>7000	ND	500-3000	1000-6000	500>7000	1000-6000
Green-dUTP	500>7000	500-6000	500-1500	200-1000	500-4000	500-5000	ND
Orange-dUTP	200-500	200-300	200-500	200-300	200-2000	200-2000	ND
Rho-5-dUTP	200-700	200-300	ND	200-300	200-500	300-2000	ND
Fl-8-dATP	500-700	200-600	ND	<200	200-500	200-500	ND
Rho-8-dATP	500-700	200-300	ND	<200	<200	200-500	ND

Sizes are given in bases.
ND means "not determined."

Example 2: In Vitro Synthesis of Fluorescent DNAs.

The fluorescent dNTPs used in these studies are shown in Table 1. The number in the abbreviated name of each compound refers to the length of the linker which joins the fluorphore to the base moiety of the dNTP.

Fluorescent dNTPs were synthesized by standard methods. The dNTPs listed as "Orange" or "Green" contain modified Rhodamine or Fluorescein dyes, respectively and are proprietary products of Imagenetics, Inc., Naperville, Ill.

The following DNA polymerases were tested: 1) T5 DNA polymerase modified to lack 3′ to 5′ exonuclease activity [T5(Exo-), BRL]. 2) T7 DNA polymerase modified to lack 3′ to 5′ exonuclease activity [T7(Exo-); U.S. Biochemical]. 3) The Klenow fragment of E. coli DNA polymerase I (BRL). 4) T. aquaticus DNA polymerase (Cetus). 5) T. litoralis DNA polymerase (Vent™, New England Biolabs). 6) Phage PRD1 DNA polymerase.

These DNA polymerases are typical of the following general types of enzymes: 1) Highly processive E. coli enzyme (T5(Exo-), T7(Exo-), Phi-29-type such as PRD-1, and E. coli pol III holozyme; 2) E. coli enzyme of low processivity (Klenow fragment); 3) Thermostable enzymes (Taq, Vent™, Vent(Exo-)™.

Prior to performing experiments with any given DNA polymerase and fluorescent dNTPs, reactions as described below were run containing increasing amounts of polymerase in the presence of the 4 normal dNTPs and a constant amount of template and primer. The amount of reaction product was quantitated by TCA precipitation of radioactive molecules. A concentration of polymerase that gave a plateau of activity in this assay was used in assays described below that test the effects of fluorescent dNTPs on synthesis. In addition, control experiments which

lacked, respectively, primer, template or single individual dNTPs (e.g., dCTP or dTTP) were run to prove that synthesis in the reactions described below was dependent on the presence of primer, template and all 4 dNTPs and thus represents copying of the DNA template by the DNA polymerase rather than some artifactual nonprimer-dependent process.

A typical reaction in which the effect, for example, of Rhodamine-8-dCTP on synthesis of DNA by T5(Exo-) DNA polymerase was determined consisted of the following:

In a total volume of 15 microliters: 27 mM Tris-HCl, pH 7.5, 13 mM $MgCl_2$, 33 mM NaCl, 7 mM dithiothreitol, 1 μg M13mp19 + Strand DNA, 5 ng 23 bp primer (BRL), 100 μM each, dATP, dGTP, dTTP, 100 to 500 μM Rho-8-dCTP and 2 μCi, ^{32}P-dGTP. The reaction was incubated at 37°C for 1 hour and 8 μl were removed. Of this, 3 μl were TCA precipitated to test for the effect of the fluorescent dNTP on activity and the remaining 5 μl were run on an alkaline agarose gel according to the procedure of Maniatis et al. (1982) "Molecular Cloning, A Laboratory Manual", first edition. Radioactive size markers (1 KB ladder, BRL) were also run on the gel to allow comparison of the apparent molecular weight of the reaction products with the standards following autoradiography of the gel. In addition, fluorescent DNA molecules in the gel could be visualized directly by placing the gel on a U.V. light transilluminator. The fluorescence pattern parallelled the radioactivity pattern. The remainder of the reaction was incubated overnight at 37°C and aliquots assayed by TCA precipitation and alkaline agarose gel electrophoresis, as above.

After a number of preliminary experiments using this protocol were performed, a standardized battery of tests was performed using this basic assay system. These experiments systematically examined the effects of the fluorescent dNTPs of Table 1 on synthesis of DNAs by the polymerases listed above. The precise buffer components and concentration of normal dNTPs

were varied to be at the optimum for each enzyme, as indicated by preliminary experiments or by data from the manufacturer. Reactions with the thermal stable polymerases, Taq, Vent™ and Vent(Exo-)™, were run at 72°C, as recommended by the manufacturer.

The reaction conditions for each of these experiments was as follows. All reactions were performed in a final volume of 15 microliters containing 1 μg M13mp19 + strand DNA, 5 ng 23 bp primer, 2 μCi ^{32}P dGTP, and, respectively in two different reactions, 100 or 500 μM of the fluorescent dNTP, and the specific buffers, concentrations of normal dNTPs and enzyme units listed below. The same results were obtained at either concentration of the fluorescent dNTP. Reactions were performed for 1 hour and overnight at the given temperature (see below) and assayed by TCA precipitation and alkaline agarose gel electrophoresis, as described above. Other aspects of the reaction conditions were:

1. For T5 (Exo-) and T7 (Exo-) DNA polymerases: Normal dNTPs (100 or 200 μM; results identical), Reaction buffer (as above), Reaction temperature (37°C), Enzyme (1 unit).

2. For Taq DNA polymerase: Normal dNTPs (200 μM), Reaction buffer (10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$) Reaction temperature (72°C), Enzyme (2 units).

3. For Vent™ and Vent (Exo-)™ polymerases: Normal dNTPs (200 μM), Reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 8 mM $Mg_2(SO_4)_2$, 0.1% Triton X-100, 100 μg/ml bovine serum albumin), Reaction temperature (72°C), Enzyme (1 unit).

4. For Klenow fragment of E. coli DNA polymerase: Normal dNTPs (100 μM), Reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 6.7 mM dithiothreitol), Reaction temperature (37°C), Enzyme (1 unit).

5. For PRD1 polymerase normal dNTP's (200μM), Reaction buffer (5mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mg/ml BSA), Reaction temperature (37°C), Enzyme (1 unit).

The most important parameter for utility of a fluorescent dNTP is the size range of products that it gives with different polymerases. These results are summarized in Table 6 for overnight enzymatic reactions.

Overnight reactions were usually performed for convenience. DNA molecules greater than 500 bases are synthesized, for example, by T5(Exo-) polymerase after 1 hour of reaction. The average size of the reaction products increases for at least 6 hours at 37°C.

Data from TCA precipitations (not shown) indicated that the various fluorescent dNTPs inhibited synthesis of DNA from ca. 40 to 99% relative to control reactions containing the 4 unmodified dNTPs. In general, those reactions that were inhibited not more than ca. 95% produced some fluorescent DNA molecules greater than 500 bases in size. There was, in general, a strong correlation between degree of inhibition and size of the fluorescent reaction products.

To determine whether two different fluorescent dNTPs could be incorporated in the same reaction, T5(Exo-) and T7(Exo-) polymerases were tested with combinations of fluorescent dNTPs, as shown in Table 6. The overnight reactions were carried out exactly as described above except that both of the two fluorescent dNTPs were present at 100 μM and the two remaining normal dNTPs were present at 200 μM concentrations. As shown in Table 6, only certain combinations of the dNTPs gave products greater than 500 bases in size.

Table 6: Size of fluorescent DNAs containing two different fluorescent labels as assayed by alkaline agarose gel electrophoresis.

	DNA Polymerase: T5(Exo-)	DNA Polymerase: T7(Exo-)
Fl-12-dUTP Rho-8-dCTP	500 - 1600*	300 - 500
Green-dUTP Rho-8-dCTP	500 - 1000	300 - 600
Fl-12-dUTP Rho-10(J)-dCTP	200 - 300	200 - 300
Fl-12-dUTP Green-dCTP	200 - 500	200 - 300
Green-dUTP Green-dCTP	200 - 300	200 - 300

* Sizes are given in bases

The following conclusions were drawn from these studies: 1) <u>Highly processive</u> DNA polymerases such as T5(Exo-), T7(Exo-) and PRD1 are particularly advantageous for synthesis of fluorescent DNAs. T5(Exo-) DNA polymerase is the best enzyme thus far examined for this purpose. 2) Fluorescent dNTPs containing linkers longer than 4 atoms (and preferably ca. 8-12 atoms) attached to non-hydrogen bonding positions are the best dNTP-Xs. 3) The chemical structure of some linkers (e.g., that of Rho-10(J)-dCTP and Fl-10(J()-dCTP) inhibits synthesis even though the linker is long. 4) The combination of Rho-8-dCTP, Fl-12-dUTP and T5(Exo-) DNA polymerase allows synthesis of fully

double nucleotide-labelled fluorescent DNAs greater than 500 bases in size.

Example 3: Stimulation of Fluorescent DNA Synthesis by Single Strand DNA Binding Protein.

The following reaction was performed. Fifteen microliter reactions, basically as described above for T5(Exo-) DNA polymerase, were performed which contained 1 unit of T7(Exo-) DNA polymerase, 0.25 or 1.0 μg of M13mp19 + strand template, respectively, 100 μM Rho-8-dCTP, 100 μM dATP, dTTP and dGTP and 6 μg of Single Strand Binding Protein (purchased from Pharmacia). The Single Strand Binding Protein enhanced synthesis of radioactive, fluorescent DNA by 205% and 168%, respectively, with the two amounts of template in a one hour reaction relative to a reaction that contained Rho-8-dCTP but no Single Strand binding Protein. Most importantly, in overnight reactions, reactions containing Single Strand Binding Protein contained fluorescent DNA of sizes 500 bp to 5 kb as compared to 500 bp to 2 kb in reactions lacking the protein. This proves that Single Strand Binding Protein can significantly enhance the synthesis of fluorescent DNA.

Example 4: Synthesis of Fluorescent Probes for In Situ Hybridization.

That probes suitable for in situ hybridization can be produced using direct incorporation of fluorescent nucleotides was shown by the following experiment. A synthesis reaction, similar to that described above, was performed using Rho-8-dCTP as the fluorescent dNTP, human Cot-1 DNA as template (Human Cot-1 DNA is total human genomic DNA greatly enriched for highly repetitive sequences and was purchased from BRL), random hexanucleotide primers (BRL) and Klenow DNA polymerase. The Human Cot-1 DNA will potentially hybridize to human DNA present in rodent-human hybrid cell lines, thus allowing the identification of the particular human chromosome contained in the rodent cell. To test this, the fluorescent DNA copy of the

human Cot-1 DNA was hybridized to metaphase spreads prepared from the WA-17 cell line, which contains human chromosome 21 in a mouse chromosome background, by standard procedures. The single copy of Human Chromosome 21 present in the cell line was readily detected by fluorescence microscopy and mouse chromosomes were not labelled by the probe. Other experiments indicated that the fluorescent Cot-1 DNA probe hybridizes to all of the human chromosome present in metaphase spreads prepared from human peripheral lymphocytes.

<u>Example 5</u>: <u>Effect of dATP Derivatives on Polymerases</u>

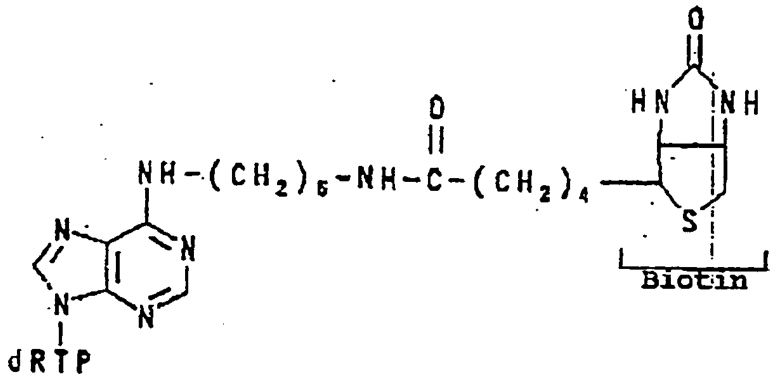
1. <u>Klenow DNA Polymerase</u>

The effect of dATP derivatives on direct incorporation by Klenow DNA polymerase was shown by the following experiment. Synthesis reactions, similar to that described in Example 2 above, were performed using normal dATP and the dATP derivatives shown in Table 7. Note that "HC-6-dUPT" and "Res-10-dUTP" refer to the commercial names for Hydroxy-coumarin-6-dUTP and Resorufin-10-dUTP, respectively, and thus the numbers in the abbreviated names do not refer to the length of the linker for these compounds.

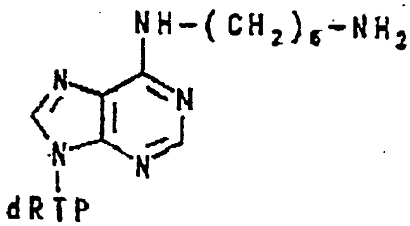
Fifteen microliter reactions were performed which contained 1 unit of Klenow <u>E</u>. <u>coli</u> DNA polymerase, 1 μ M13mp19 + Strand DNA, 5 ng 23 pb primer (BRL), 100 μM each dCTP, dGTP and dTTP, reaction buffer, 2 μCi ^{32}P-dGTP, and, respectively in two different reactions, 25 or 100 μM of the normal dATP or dATP derivative. Reactions were performed for 1 hour at the 37° C and assayed by TCA precipitation and alkaline agarose gel electrophoresis, as described above. The results are summarized in Table 8. As shown by the percent activities, both bio-7-dATP and AH-dATP are better substrates than the fluorescent dATPs, and both produce larger reaction products. Reactions containing bio-7-dATP produced DNA of sizes 1000-1600 bases and AH-dATP (linker only) produced DNA of sizes 500-2000 bases; reactions containing fluorescent dATPs produced fluorescent DNA of sizes less than 500

bases. This proves that incorporation of biotin dATP or AH-ATP is not predictive of the incorporation of the cognate fluorescent dATPs, nor does synthesis of biotinylated DNA predict the synthesis of large fluorescent DNAs.

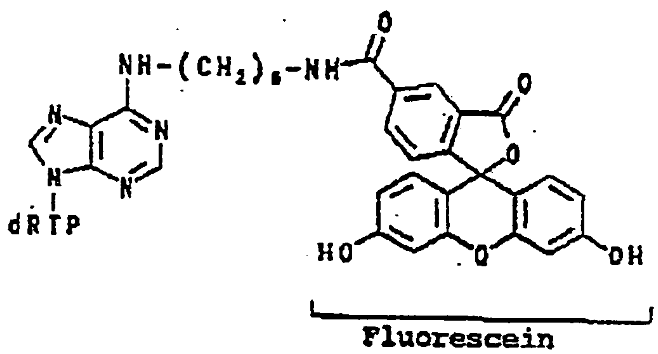
TABLE 7



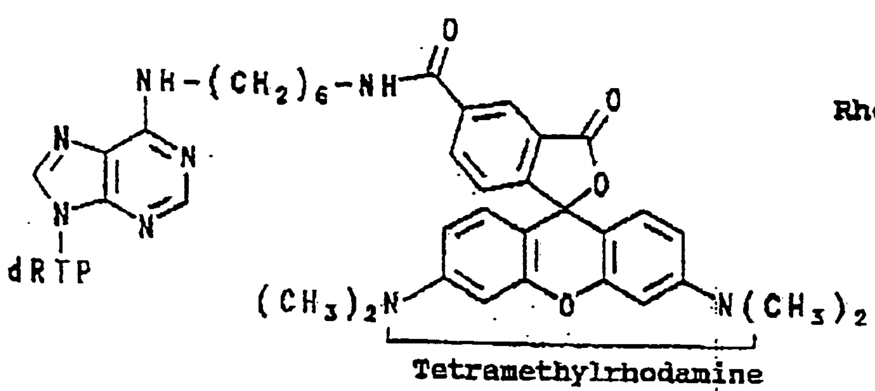
Bio-7-dATP



AH-dATP



Fl-7-dATP



Rho-7-dATP

TABLE 8. EFFECT OF dATP DERIVATIVES ON KLENOW DNA POLYMERASE

	25 μM (dATP Derivative Conc.)			100 μM (dATP Derivative Conc.)		
dATP Derivative	CPM Incorp.	% Activity	Size of Products	CPM Incorp.	% Activity	Size of Products
dATP	5998	100	1000-4000	20064	100	1600->7000
Bio-7-dATP	640	10.7	1000-1500	680	3.4	1000-1600
AH-dATP	1277	21.3	500-1200	1831	9.1	1000-2000
Fl-7-dATP	456	7.6	<500	410	2.0	<500
Rho-7-dATP	0	0	---	377	1.9	<500

Sizes are given in bases.
CPM means "counts per minute".
% Activity based on 100% for normal dATP

1. <u>T5(Exo-) DNA Polymerase</u>

The effect of dATP derivatives on direct incorporation by T5(Exo-) DNA polymerase was shown by the following experiment. The 1 hour reactions were carried out exactly as described above except that T5(Exo-) DNA polymerase was used. The results are summarized in Table 9.

TABLE 9. EFFECT OF dATP DERIVATIVES ON T5(Exo-) DNA POLYMERASE

	25 μM (dATP Derivative Conc.)			100 μM (dATP Derivative Conc.)		
dATP Derivative	CPM Incorp.	% Activity	Size of Products	CPM Incorp.	% Activity	Size of Products
dATP	3970	100	1000-4000	14464	100	2000->7000
Bio-7-dATP	2026	51	1000-1600	3130	21.6	1000-1600
AH-dATP	1295	32.6	1000-1600	5447	27.7	1600-6000
Fl-7-dATP	410	10.3	<500	0	0	---
Rho-7-dATP	33	0.8	<500	3563	24.6	<500

Sizes are given in bases.
CPM means "counts per minute".
% Activity based on 100% for normal dATP.

The following conclusions were drawn from these studies: 1) T5(Exo-) DNA polymerase is a better enzyme for incorporating biotin dATP and AH-ATP than is the Klenow polymerase, as shown by the percent activities. 2) Rho-7-dATP at a high concentration is a much better substrate for T5(Exo-) polymerase (24.6% activity) than it is for Klenow polymerase (1.9% activity). 3) Both bio-7-dATP and AH-dATP produce larger reaction products than fluorescent dATPs, which produced fluorescent DNA of sizes less than 500 bases. 4) Incorporation of biotin dNTP or AH-NTP by a particular polymerase is not predictive of the incorporation of the cognate fluorescent dNTPs by that same polymerase. 5) Incorporation of biotin dNTP or AH-NTP does not predict the differences in incorporation of different fluorescent dNTPs by different polymerases (see also Table 5 above). 6) Synthesis of biotinylated DNA does not predict the synthesis of large fluorescent DNAs. 7) A given fluorescent dNTP is both an inhibitor and a substrate for DNA polymerases, and different polymerases thus have different Km and Ki values for a given dNTP. Differences in Km and/or Ki values is shown, for example, by comparing the percent activities of Rho-7-dATP and Fl-7-dATP. Rho-7-dATP is a much better substrate for the T5(Exo-) polymerase as the concentration is raised from 25 to 100 μM; the activity of Fl-7-dATP, on the other hand, decreases with increasing concentration. 8) The fact that different polymerases have different Km and Ki values for fluorescent dNTPs makes it impossible to predict a priori the activity of a given fluorescent dNTP with a given polymerase. The behavior of fluorescent dNTPs with polymerases cannot be predicted from the behavior of biotinylated nucleotides, for example, since biotinylated nucleotides themselves have different effects on Km and Ki values.

Example 6: Synthesis of Fluorescent DNAs

The effect of several modified dNTPs on direct incorporation by various polymerases, including reverse transcriptases and highly processive enzymes, was shown by the following experiment. The fluorescent dNTPs used in these studies are shown in Tables 1 and 2. Res-10-dUTP (Resorufin-10-dUTP) and HC-6-dUTP (Hydroxy-coumarin-6-dUTP) were purchased from Boehringer Mannheim Biochemica. Fl-12-dUTP, Rho-12-dUTP, Rho-12-dUTP (R) and Rho-8-dCTP were synthesized as described in Example 1. Rho-12-dUTP and Rho-12-dUTP (R) differ in the structure of the linker, the latter compound having a more rigid linker than the former. The reaction conditions were identical to those of Example 2 except that the reactions were performed overnight with one of the following polymerases: T5(Exo-), purchased from BRL; T7(Exo-), purchased from U.S. Biochemical; AMV-RT and M-MLV-RT, purchased from BRL; and HIV-RT, obtained from Dr. Steven Hughes of the National Cancer Institute. The three reverse transcriptases (AMV-RT, M-MLV-RT and HIV-RT) copied DNA templates and thus were used as DNA polymerases. Reactions were assayed by TCA precipitation and alkaline agarose gel electrophoresis, as described above. The results are summarized in Table 10.

TABLE 10. SIZE OF FLUORESCENT DNA's SYNTHESIZED IN OVERNIGHT REACTIONS AS ASSAYED BY ALKALINE AGAROSE GEL ELECTROPHORESIS.

dNTP	T5(Exo-)	T7 (Exo-)	AMV-RT	HIV-RT	M-MLV-RT
Res-10-dUTP	500-2000	200-800	<200	<200	400-600
HC-6-dUTP	500->7000	500->7000	500-2000	500-1000	500-3000
Rho-12-dUTP	500-2000	300-3000	500-2000	300-2000	500-1000
Rho-12-dUTP (R)	800->7000	300-3000	500-1000	<200	<200
Fl-12-dUTP	ND	ND	400-1200	400-600	300
Rho-8-dCTP	ND	ND	<300	<300	<300

Sizes are given in bases.
ND means "not determined".

The following conclusions were drawn from these studies: 1) HC-6-dUTP is a particularly good substrate with T5(Exo-) and T7(Exo-) polymerases and promotes the synthesis of large fluorescent DNAs (some greater than 7 kb in size). 2) The rigid linker of Rho-12-dUTP (R) is particularly advantageous for T5(Exo-) DNA polymerase relative to Rho-12-dUTP. 3) The reverse transcriptases synthesize fluorescent DNAs greater than 200 bases in size using certain dNTPs, particularly HC-6-dUTP and Rho-12-dUTP. Like other polymerases, the reverse transcriptases also vary in their abilities to incorporate certain fluorescent dNTPs (see above).

CLAIMS

1. A method of making fluorescence-labelled nucleic acid in an enzyme catalyzed reaction comprising the steps of:

   a. combining template nucleic acid, a nucleic acid synthesis-catalyzing enzyme, a ribo- or deoxynucleotide mixture comprising a ribo- or deoxynucleotide selected from the group r- or dATP or r- or dATP-X, a ribo- or deoxynucleotide selected from the group r- or dGTP or r- or dGTP-X, a ribo- or deoxynucleotide selected from the group r- or dCTP or r- or dCTP-X and a ribo- or deoxynucleotide selected from the group dTTP, r- or dUTP, dTTP-X or r- or dUTP-X, wherein X is a fluorophore covalently linked by a linkage group to said ribo- or deoxynucleotide, said linkage group having a linear chain of greater than 7 atoms length, and wherein said ribo- or deoxynucleotide mixture comprises at least one of r- or dATP-X, r- or dGTP-X, r- or dUTP-X, dTTP-X or r- or dCTP-X, and a diluent compatible with DNA-synthesizing enzyme activity, to form a reaction mixture, all ribo- or deoxynucleotides in the reaction mixture being either ribo- or deoxynucleotides; and

   b. incubating said reaction mixture for a time sufficient to synthesize a detectable amount of fluorescent-labelled nucleic acid having at least 200 bases length.

2. The method of claim 1 wherein the DNA synthesizing enzyme is a DNA polymerase.

3. The method of claim 1 wherein the DNA synthesizing enzyme is T5, T7, or PRD1 DNA polymerase.

4. The method of claim 1 wherein the DNA synthesizing enzyme is reverse transcriptase.

5. The method of claim 1 wherein the reaction mixture additionally includes a single strand binding protein.

6. The method of claim 1 wherein the enzyme is an RNA polymerase.

7. The method of claim 1 wherein the enzyme is an RNA replicase.

8. The method of claim 1 wherein the enzyme is T5 or T7 polymerase, dCTP-X is Rho-8-dCTP and the reaction mixture further comprises a single-strand binding protein.

9. The method of claim 1 wherein the enzyme is T5 or T7 polymerase, dUTP-X is Rho-12-dUTP and the reaction mixture further comprises single-strand binding protein.

10. The method of claim 1 wherein the enzyme is Vent™ polymerase dUTP-X is Rho-12-dUTP and the reaction mixture further comprises a single-strand binding protein.

11. The method of claim 1 wherein dCTP-X is Rho-8-dCTP, Fl-8-dCTP, Fl-15-dCTP or Rho-15-dCTP.

12. The method of claim 1 wherein dUTP-X is Fl-12-dUTP, Rho-12-dUTP or Green-dUTP.

13. The method of claim 1 wherein the fluorescent-labelled nucleic acid has at least 500 bases length.

14. The method of claim 1 wherein said linkage group lacks an ether linkage.

15. The method of claim 1 wherein said linkage group has a linear chain of 8-12 atoms length.

16. A method for synthesizing DNA in an enzyme catalyzed reaction, wherein said method comprises combining a DNA polymerase, a DNA template, a primer and 4 dNTPs, the improvement comprising completely substituting for at least one of the dNTPs a fluorescence-labelled dNTP selected from dATP-X, dGTP-X, dCTP-X, dTTP-X or dUTP-X, wherein X is a fluorophore covalently linked by a linkage group of greater than 7 atoms length to a nonhydrogen-bonding site of said dNTP, said improvement resulting in fluorescence-labelled DNA having at least 200 bases length.

17. The method of claim 16 wherein the fluorescence-labelled DNA has at least 500 bases length.

18. The method of claim 16 wherein the linkage group of the fluorescence-labelled dNTP lacks an ether group.

19. The method of claim 16 wherein the reaction mixture further comprises single strand DNA binding protein.

20. The method of claim 16 wherein the linkage group is of 8-12 atoms length.

21. The method of claim 16 wherein more than one of the dNTP's is completely substituted by a fluorescence labelled dNTP.

22. The method of claim 16 wherein the DNA polymerase is T5 polymerase and the reaction mixture comprises Fl-12-dUTP, Rho-8-dCTP, dATP and dGTP.

23. The method of claim 16 wherein the DNA polymerase is T5 polymerase, and the reaction mixture comprises Green-dUTP, Rho-8-dCTP, dATP and dGTP.

24. Fully single nucleotide labelled DNA of greater than 500 nucleotides length comprising 3 deoxynucleotides and one labelled deoxynucleotide selected from the group Rho-8-dCTP, Fl-15-dCTP, Rho-15-dCTP, Rho-12-dUTP, Green-dUTP, Orange-dUTP, Fl-8-dATP, or Rho-8-dATP.

25. Fully double nucleotide labelled DNA comprising two deoxynucleotides and two labelled deoxynucleotides.

26. Fully double nucleotide labelled DNA according to claim 25, wherein the first labelled deoxynucleotide is selected from the group Fl-12-dUTP or Green-dUTP, and the second labelled deoxynucleotide is selected from the group Rho-8-dCTP, Rho-10(J)-dCTP or Green-dCTP.

27. The compound Rho-12-dUTP.

28. A dCTP-X selected from the group Rho-8-dCTP, Fl-8-dCTP, Rho-15-dCTP and Fl-15-dCTP.

29. A dATP-X selected from the group Rho-8-dATP, Fl-8-dATP, and Fl-15-dATP.

30. A kit for making a fluorescent nucleic acid having four ribo- or deoxynucleotides in its composition and having at least one of the ribo- or deoxynucleotides completely substituted by a fluorescent labelled ribo- or deoxynucleotide comprising:

a) a nucleic acid synthesizing enzyme;

b) a mixture of ribo- or deoxynucleotide triphosphates comprising not more than three r- or dNTP's selected either from the group rATP, rGTP, and rYTP, wherein rYTP is rCTP, rTTP or rUTP, or the group dATP, dGTP, and dYTP, wherein dYTP is dCTP, dTTP or dUTP, and at least an r- or dNTP-X selected either from the group

rATP-X, rGPT-X, and rTYP-X, wherein rYTP-X is rCTP-X, rTTP-X or rUTP-X, or the group dATP-X, dGTP-X, and dYTP-X, wherein dYTP-X is dCTP-X, dTTP-X or dUTP-X such that no selected r- or dNTP-X is a derivative of any selected r- or dNTP, the selected r- or dNTP's being present in non-limiting concentrations for enzyme-catalyzed nucleic acid synthesis; and

c) reaction buffer and components sufficient to provide conditions suitable for activity of the nucleic acid synthesizing enzyme.

31. A kit for making a fluorescent nucleic acid having four ribo- or deoxynucleotides in its composition and having at least one of the ribo- or deoxynucleotides completely or partially substituted by a fluorescent labelled ribo- or deoxynucleotide comprising:

a) a nucleic acid synthesizing enzyme;
b) four r- or dNTP's;

c) a dNTP-X selected from the group Rho-12-r- or dUTP, Rho-8-r- or dCTP, Fl-8-r- or dCTP, Rho-15-r- or dCTP, Fl-15-r- or dCTP, Rho-8-r- or dATP, Fl-8-r- or dATP, and Fl-15-r- or dATP.

32. The method of claim 1 wherein the DNA synthesizing enzyme is a highly processive enzyme.

33. The method of claim 4 wherein the reverse transcriptase is AMV-RT, M-MLV-RT or HIV-RT.

34. The method of claim 32 wherein the highly processive enzyme is a T5 polymerase and derivatives thereof.

35. The method of claim 32 wherein the highly processive enzyme is a T7 polymerase and derivatives thereof, and wherein the reaction mixture further comprises a thioredoxin cofactor.

36. The method of claim 32 wherein the highly processive enzyme is a Phi-29-type DNA polymerase.

37. The method of claim 32 wherein the highly processive enzyme is PRD1 polymerase.

38. The method of claim 32 wherein the highly processive enzyme is E. coli pol III holoenzyme.

39. A method of making fluorescence-labelled nucleic acid in an enzyme catalyzed reaction comprising the steps of:

a. combining template nucleic acid, a nucleic acid synthesis-catalyzing enzyme, a ribo- or deoxynucleotide mixture comprising a ribo- or deoxynucleotide selected from the group r- or dATP or r- or dATP-X, a ribo- or deoxynucleotide selected from the group r- or dGTP or r- or dGTP-X, a ribo- or deoxynucleotide selected from the group r- or dCTP or r- or dCTP-X and a ribo- or deoxynucleotide selected from the group dTTP, r- or dUTP, dTTP-X or r- or dUTP-X, wherein X is a fluorophore covalently linked by a linkage group to said ribo- or deoxynucleotide, said linkage group having a linear chain of at least 5 atoms length, and wherein said ribo- or deoxynucleotide mixture comprises at least one of r- or dATP-X, r- or dGTP-X, r- or dUTP-X, dTTP-X or r- or dCTP-X, and a diluent compatible with DNA-synthesizing enzyme activity, to form a reaction mixture, all ribo- or deoxynucleotides in the reaction mixture being either ribo- or deoxynucleotides; and

b. incubating said reaction mixture for a time sufficient to synthesize a detectable amount of fluorescent-labelled nucleic acid having at least 200 bases length.

40. The method of claim 39 wherein the fluorescent-labelled nucleic acid has at least 500 bases length.

41. The method of claim 39 wherein dUTP-X is HC-6-dUTP, Res-10-dUTP or Rho-12-dUTP (R).

42. The method of claim 39 wherein dUTP-X is HC-6-dUTP and the enzyme is T5 or T7.

43. The method of claim 39 wherein dUTP-X is HC-6-dUTP and the enzyme is selected from the group consisting of AMV-RT, HIV-RT and M-MLV-RT.

44. The method of claim 39 wherein dUTP-X is Res-10-dUTP and the enzyme is T5.

45. The method of claim 39 wherein dUTP-X is Rho-12-dUTP (R) and the enzyme is T5.

46. The method of claim 39 wherein the DNA synthesizing enzyme is AMV-RT, M-MLV-RT or HIV-RT.

47. A method for synthesizing DNA in an enzyme catalyzed reaction, wherein said method comprises combining a DNA polymerase, a DNA template, a primer and 4 dNTPs, the improvement comprising completely substituting for at least one of the dNTPs a fluorescence-labelled dNTP selected from dATP-X, dGTP-X, dCTP-X, dTTP-X or dUTP-X, wherein X is a fluorophore covalently linked by a linkage group of at least 5 atoms length to a nonhydrogen-bonding site of said dNTP, said improvement resulting in fluorescence-labelled DNA having at least 200 bases length.

48. The method of claim 47 wherein the fluorescence-labelled DNA has at least 500 bases length.

49. The method of claim 47 wherein dUTP-X is HC-6-dUTP, Res-10-dUTP or Rho-12-dUTP (R).

50. The method of claim 47 wherein dUTP-X is HC-6-dUTP and the enzyme is T5 or T7.

51. The method of claim 47 wherein dUTP-X is HC-6-dUTP and the enzyme is selected from the group consisting of AMV-RT, HIV-RT and M-MLV-RT.

52. The method of claim 47 wherein dUTP-X is Res-10-dUTP and the enzyme is T5.

53. The method of claim 47 wherein dUTP-X is Rho-12-dUTP (R) and the enzyme is T5.

54. The method of claim 47 wherein the DNA synthesizing enzyme is AMV-RT, M-MLV-RT or HIV-RT.

55. Fully single nucleotide labelled DNA of greater than 500 nucleotides length comprising 3 deoxynucleotides and one labelled deoxynucleotide selected from the group consisting of HC-6-dUTP, Res-10-dUTP and Rho-12-dUTP (R).

56. The compound Rho-12-dUTP (R).

57. A kit for making a fluorescent nucleic acid having four ribo- or deoxynucleotides in its composition and having at least one of the ribo- or deoxynucleotides completely or partially substituted by a fluorescent labelled ribo- or deoxynucleotide comprising:

a) a nucleic acid synthesizing enzyme;

b) four r- or dNTP's;

c) a dNTP-X selected from the group consisting of Rho-12-r- or dUTP (R), HC-6-r- or dUTP, and Res-10-r or dUTP.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02422

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34; C07H 21/04
US CL :435/6, 91; 536/23.1, 25.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/23.1, 25.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WORLD PATENTS, CA, BIOTECHNOLOGY ABSTRACTS
search terms: fluorescent-labelled DNA, DNA sequencing, polymerase chain reaction, fluorescent-labelled nucleotides

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Methods in Molecular and Cellular Biology, Volume 3, issued February 1992, H. Voss et al, "New Procedure for Automated DNA Sequencing with Multiple Internal Labeling by Fluorescent dUTP", pages 30-34, especially page 33.	1-4,12,13, 15-17,20,34, 35,39,40,47, 48,54 5-11, 14, 18,19,21-26, 30-33,36-38,41-46,49-53,55,57
Y	Journal of Cellular Biochemistry, Supplement 16B, issued 1992, M. L. Hammond et al, "Enzymatic Synthesis and Exonucleolytic Degredation of Fluorescent DNA Containing Rhodamine and Fluorescein Nucleotides", see Abstract F325.	1-57

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 APRIL 1993	04 MAY 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CARLA MYERS Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BioTechniques, Volume 8, No. 6, issued 1990, J.J. Lanzillo, "Preparation of Digoxigenin-Labelled Probes by the Polymerase Chain Reaction, pages620-622, see entire document.	1-26, 30-55, 57
Y	Technique, Volume 2, No. 3, issued June 1990, D. Pollard-Knight, "Current Methods in Nonradioactive Nucleic Acid Labeling and Detection, pages 113-132, especially pages 115-119.	1-26, 30-55, 57
Y	Science, Volume 238, issued 16 October 1987, J. M. Prober et al, "A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides", pages 336-340, especially page 337.	1-26, 30-55, 57
Y	Nucleic Acids Research, Volume 18, No. 4, issued February 1990, K. Schwarz et al, "Improved Yields of Long PCR Products Using Gene 32 Protein", page 1079, see entire document.	5, 8-10, 19
Y	Pharmacia LKB Biotechnology Products Catalog, issued 1989, page 32.	5, 8-10, 19
Y	Proceedings National Academy of Sciences, USA, Volume 85, issued August 1988, J. A. Brumbaugh et al, "Continuous, on-line DNA Sequencing using oligodeoxynucleotide primers with multiple fluorophores", pages 5610-5614, especially figure 1.	8,9,11,12, 14,27,30,31
Y	Analytical Biochemistry, Volume 193, issued 1991, A. Landgraf et al, "Quantitative Analysis of Polymerase Chain Reaction (PCR) Products Using Primers Labeled with Biotin and a Fluorescent Dye", pages 231-235, see entire document.	8,9,11,30, 31,41,45, 49,53,55,57
Y	Tetrahedron, Volume 43, No. 15, issued 1987, S.R. Sarfati et al, "Synthesis of Fluorescent or Biotinylated Nucleoside Compounds", pages 3491-3497, especially pages 3492-3493.	28,29
X,P Y	Electrophoresis, Volume 13, issued September 1992, W. Ansorge et al, "High-Throughput Automated DNA Sequencing Facility with Fluorescent Labels at the European Molecular Biology Laboratory", pages 616-619, see entire document.	1-3,12,13-18,29,32,39,40,42,47,48 4-11, 19-28, 30,31,33-38, 41,43,44-46, 49-57